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(71) Applicant: ETHIGEN CORPORATION [US/US]; 6320 Commodore Sloat Drive, Los Angeles, CA 70048 (US).

(72) Inventor: LIPPA, Arnold ; 2500 Hudson Terrace, Fort Lee, NJ 07024 (US).

(74) Agents: FIGG, E., Anthony et al.; Bernard, Rothwell & Brown, 1700 K Street NW, Suite 800, Washington, DC 20006 (US).

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(54) Title: FLUORESCENCE IMMUNOASSAY INVOLVING ENERGY TRANSFER BETWEEN TWO FLUOROPHORES

(57) Abstract

In a fluorescence immunoassay system, first and second fluorophores are covalently bound to one of a member of a specific binding pair of ligand and receptor, the first of said fluorophores being capable of absorbing light at a first wavelength to produce light emission at a second wavelength, which second wavelength can be absorbed by the second fluorophore.

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FLUORESCENCE IMMUNOASSAY INVOLVING ENERGY TRANSFER BETWEEN TWO FLUOROPHORES.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of fluorescence immunoassays useful in the determination of analytes in fluid samples.

Description of the Background Art

There are several known methods for detecting analytes (e.g., hormones, enzymes, other proteins, therapeutic agents, drugs of abuse, etc.) in liquid samples such as biological fluids. Among the known types of methods, immunoassays have emerged as sensitive techniques for determining minute amounts of certain organic compounds. Immunoassay methods generally are based on the ability of a receptor molecule, usually an antibody, to specifically recognize a particular spatial and/or polar organization of a ligand molecule, and thereby selectively bind to the ligand molecule.

Certain of the known immunoassay techniques involve the use of fluorophore molecules, which are able to absorb light a one wavelength and emit light at another wavelength. For example, U.S. Patent No. 4,272,505 to Smith describes a method for assaying a biological fluid sample for a thyroid hormone. This method is based on the principle of fluorescence suppression of a fluorophore by thyroid hormone. The Smith method is a competitive-type assay involving formation of a mixture of a fluid sample with a known amount of fluorophore-labeled thyroid hormone, the fluorophore having a fluorescence level which is substantially suppressed by the thyroid hormone to which the fluorophore is bound. Antibody capable of binding to the fluorophore-labeled thyroid hormone as well as thyroid hormone present in the sample is introduced into the mixture. The antibody is thought to sterically alter the labeled thyroid hormone,

thus changing the degree of suppression of the fluorescence of the fluorophore bound thereto. The fluorescence level of the mixture then is measured and the amount of thyroid hormone in the sample is calculated by comparing the fluorescence level of the mixture with a standard fluorescence level.

U.S. Patent No. 4,133,873 to Noller discloses a method for determining the amount of a member of a gr up consisting of an extracellular antigen and an extracellular antibody capable of specifically combining with said antigen. The method involves tagging the member with a fluorophore and exposing the tagged member to a pulse of light of a first wavelength sufficient to cause emission by the tagged exposed member of secondary light having a second wavelength different from the first wavelength. The secondary light is sensed to generate a perceptible signal in response to and commensurate with the sensed secondary light.

An immunoassay utilizing two different ligands tagged with separate fluorophores which independently fluoresce at different wavelengths is disclosed in U.S. Patent No. 4,385,126 to Chen et al. The two tagged ligands are capable of immunologically binding to each other, and the two different ligands may be detected independently through their independent tagging constituents (fluorophores) for quality control, internal calibration (standardization), determination of viability and shelf life, and the like.

U.S. Patent Nos. 3,996,345, 4,174,384, 4,199,559 and 4,261,968, all to Ullman et al., disclose immunoassays employing antibodies and a fluorescer-quencher chromophoric pair. The methods are based on the phenomenon of energy transfer between two chromophores which form a fluorescer-quencher pair. The methods involve irradiation of a fluorescer molecule with light

of a first wavelength which is absorbed by the fluorescer and resultant emission f light of a longer wavelength by the fluorescer. If a quencher chromophore is within less than about 100 Å of the fluorescer and absorbs light at the wavelength of emission of the fluorescer, the fluorescer will transfer to the quencher chromophore the energy which would otherwise have been emitted as light. The Ullman methods all involve measurement of the decrease of fluorescence (light emission) of the fluorescer chromophore resulting from energy transfer to the quencher chromophore. Ligand and antiligand can be separately labeled with fluorescer and quencher, or one group of antibodies can be labeled with fluorescer and another group of antibodies labeled with quencher for detection of ligand capable of immunologically binding to more than one antibody.

U.S. Patent No. 4,536,479 to Vander-Mallie discloses an immunoassay method for the detection of an analyte in a test sample wherein a reaction mixture is formed between test sample and a pair of reagents. The first reagent is an idiotypic anti-analyte antibody labeled with a first fluorophore. The second reagent is an anti-idiotypic antibody labeled with a second fluorophore, which anti-idiotypic antibody is capable of competing with analyte in the sample for the idiotypic anti-analyte antibody. One of the fluorophores is capable of absorbing incident light at a first wavelength to produce light emission at a second wavelength which second wavelength can be absorbed by the other fluorophore to produce emission at a third wavelength. For the detection of analyte in the reaction mixture, the reaction mixture is irradiated with incident light of the first wavelength, and the intensity of light of the second or third wavelength is measured, which intensity

is related to the amount of analyte initially present in the test sample.

All of the known fluorescence immunoassay methods have disadvantages and limitations, leaving a continuing need for new, rapid and sensitive methods for detecting analytes in fluid samples.

SUMMARY OF THE INVENTION

In accordance with the present invention, a member of a specific binding pair of ligand and receptor is provided, the member being covalently bound to first and second fluorophores. The first of the fluorophores is capable of absorbing light at a first wavelength to produce light emission at a second wavelength, which second wavelength can be absorbed by the second fluorophore. The method of the invention utilizes a member of a specific binding pair as defined above in the determination of the presence or amount of the other member of the specific binding pair in a fluid sample.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic depiction showing reduction of fluorescence excitation transfer between fluorescein and eosin bound to anti-thyroxine (anti-T4) in the presence of varying concentrations of thyroxine (T4).

FIG. 2 is a graphic depiction showing the effect of cold T4 on the relative fluorescence of double-labeled antibody to T4.

FIG. 3 is a graphic depiction showing the effect of volume of Hepatitis B Surface Antigen on the fluorescence of monoclonal antibody to Hepatitis B labeled with fluorescein isothiocynate and eosin.

FIG. 4 is a graphic depiction showing the effect of volume of immunoglobulin G (IgG) on the fluorescence of antibody to IgG labeled with fluorescein isothiocynate and eosin.

FIG. 5 is a graphic depiction showing an increase in fluorescence excitation transfer upon incubation of double-labeled digoxin antibody (anti-DG) with varying concentrations of digoxin (DG).

FIG. 6 is a graphic depiction showing the effect of cold DG on the fluorescence of anti-DG double labeled by fluorescein isothiocynate (FTIC) and eosin.

FIG. 7 is a graphic depiction showing the effect of normal serum (diluted 1:10) on fluorescence of double-labeled monoclonal anti-IgG.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In carrying out an immunoassay in accordance with the present invention, a member of a specific binding pair of ligand and receptor is employed, which member is covalently bound to first and second fluorophores. The first and second fluorophores can be covalently bound to either the ligand or receptor member of the specific binding pair for detection of the other member of the specific binding pair.

Ligand refers to an organic molecule or assemblage with at least one functionality having a particular spatial and/or polar organization for which a receptor is either naturally available or can be prepared.

Receptor refers to a molecule which is capable of specifically recognizing a certain functionality of a ligand molecule having a particular spatial and/or polar organization, and thereby selectively binding to the ligand molecule. Receptors generally are antibodies although enzymes, proteins, nucleic acids, and certain globulins, may also act as receptors.

A fluorophore is a molecule capable of absorbing light at one wavelength and emitting light at another wavelength.

In accordance with the invention, the first and second fluorophores covalently bound to ligand or

receptor are members of a fluorescence energy transfer system pair where the emission spectrum of the first fluorophore overlaps well with the excitation spectrum of the other fluorophore such that when the first and second fluorophores interact by fluorescence energy transfer, the fluorescence of the first fluorophore is absorbed by the second fluorophore by energy transfer.

Examples of fluorophores having overlapping emission and excitation spectrums include fluorescein (excitation λ 495 nm, emission λ 520 nm), eosin (excitation λ 520 nm, emission λ 545 nm), fluorescamine (excitation λ 390 nm, emission λ 520 nm), and tetramethyl rhodamine (excitation λ 520 nm, emission λ 550 nm).

According to one embodiment, the fluorescence energy transfer system pair includes fluorescein as a first fluorophore and eosin as a second fluorophore.

In accordance with one embodiment, first and second fluorophores are brought within excitation transfer proximity by covalently binding both chromophores to one member of a specific binding pair of ligand and receptor.

The first and second fluorophores are covalently bound to ligand or receptor in sufficiently close proximity to each other (generally less than about 100 A) such that an energy transfer from the first fluorophore to the second fluorophore will take place upon excitation of the first fluorophore by light of a first (excitation) wavelength. Alternatively, the bound fluorophores are proximally positioned by binding of ligand and receptor.

The non-covalent attachment of the fluorophore-labeled member of the specific binding pair to the unlabeled member may either interfere with or enhance the fluorescence excitation transfer resulting in either a quenching or enhancement of fluorescence of one or both fluorophores.

The fluorophores can be covalently bound to ligand or receptor using any suitable method known in the art. For example, if receptor is antibody, the first and second fluorophores generally are sequentially bound to the antibody. Antibodies generally have a number of active amino groups which can be used for covalently binding fluorophores to the antibody. Conveniently, a fluorophore can have a non-oxocarbonyl functionality (including the nitrogen and sulfur analogs thereof) or active α-halocarbonyl functionality. Illustrative functionalities for linking a fluorophore to antibody include acylhalides, mixed anhydrides, imidate alkyl esters, isothiocynate, chlorobromo- or iodoacetyl, and the like.

The conditions for covalent bonding employ moderate temperatures, e.g., 0°-40°C, in aqueous media at moderate pH. Covalent bonding of fluorophores to protein is known in the art, see, e.g., The et al., Immunology, 18:865 (1970); Cebra et al., J. Immunol., 95:230 (1965); Goldman, Fluoroescence Antibody Methods, Academic Press, New York (1968). Energy transfer between a fluorescence energy donor (such as fluorescein) in a suitable energy acceptor (such as eosin) depends on the inverse sixth power of the distance between donor and acceptor as well as the dielectric constant of the immediate environment. Energy transfer is generally most efficient over a distance on the order of 40-50 Å, such that a fluorescence energy donor transfers energy to a nearest adjacent acceptor rather than a more distant one.

The invention will further be specifically described with respect to the first and second fluorophores covalently bound to receptor (antibody) although it is to be understood that the invention is equally applicable to ligand-bound fluorophore pairs.

With first and second fluorophores proximally bound to antibody absorption of light by the first fluorophore at a first excitation wavelength (λ_{ex_1}) produces light emission at a second emission wavelength (λ_{em_1}), and the second wavelength (λ_{em_1}) can be absorbed by the second fluorophore to produce an emission at a third wavelength (λ_{em_2}).

Binding of labeled antibody to ligand inhibits energy transfer from the first fluorophore to the second fluorophore, and thus increases fluorescence of the first fluorophore (i.e., increases emission of light wavelength λ_{em_1}), and correspondingly decreases fluorescence of the second fluorophore (i.e., emission of light wavelength λ_{em_2}) due to the inhibition of energy transfer between the first fluorophore and the second fluorophore by the bound ligand molecule.

Fluorophore-labeled antibody to ligand is utilized to determine the presence or amount of ligand in a fluid sample. A reaction mixture is formed by contacting the fluid sample with double fluorophore-labeled antibody, and specific binding of labeled antibody and unlabeled ligand inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

Inhibition of energy transfer between the first and second fluorophores upon irradiation of the reaction mixture with light of a first excitation wavelength (λ_{ex_1}) is a function of the amount of unlabeled analyte (unlabeled ligand) present in the fluid sample. The quantity of unlabeled ligand can be determined by irradiating the reaction mixture with light of a first excitation wavelength (λ_{ex_1}) and measuring the amount of fluorescence from the reaction mixture of light of wavelength λ_{em_1} or λ_{em_2}, which fluorescence is directly related to the amount of analyte (unlabeled ligand) present in the fluid sample.

The method of the present invention can be carried out either by measuring the increase of λ_{em1} emission due to receptor-ligand binding as compared to a standard containing labeled antibody in the absence of ligand, or measuring the decrease in λ_{em2} emission as a result of antibody/ligand binding as compared to a standard containing like amount of labeled antibody in the absence of ligand.

The exact mechanism of the excitation energy transfer changes brought about by binding of ligand and antibody is not known, and may be due to differing factors depending upon the ligand/receptor pair used. Without being bound to any particular theory, such mechanisms may include steric or physical hinderance by the bound ligand of energy transfer between the first and second fluorophores, or other changes in the micro-environment of the bound ligand/receptor pair causing an emission change.

Non-limiting examples of ligand/receptor pairs with which the invention has heretofore been practiced include hepatitis B surface antigen and antibody thereto, immunoglobulin G and antibody thereto, thyroxine and antibody thereto, and digoxin and antibody thereto.

The present invention provides a specific and sensitive non-competitive immunoassay having the benefits of simplicity and requiring fewer steps than prior art assays. The immunoassay of this invention is also more economical than prior art assays, requiring fewer reagents.

The invention is further illustrated by the following examples which are not intended to be limiting.

EXAMPLE I

Thyroxine (T4) Assay

1. Preparation of Fluorescein Labeled T4 Antibody (T4 AB)

Fluorescein labeled T4 AB was obtained by reaction of 1 volume of 20 g/l of fluorescein isothiocynate (FTIC) with 2 volumes of 20 g/l T4 AB (Calbiochem-Behring) in a pyridine/water/triethyl-amine medium of composition 9:1.5:0.1 v/v/v. Reaction was complete after 1 hour at room temperature. The excess of unreacted fluorescein was removed by dialysis against 5 mM K-phosphate buffer pH 7.35 containing 150 mM NaCl. Overnight dialysis was sufficient to remove unreacted material.

2. Preparation of Double Labeled T4 AB

Eosin-maleimide was obtained from Molecular Probes, Inc. Double-labeled T4 AB was prepared by incubating fluorosceine-labeled T4 AB with eosin maleimide (20 x molar excess) in 20 mM histidine-HCl buffer (pH 7.40) for 3 hours at 20-25 C. The excess of unreacted eosin was removed by dialysis against 5 mM K-phosphate buffer pH 7.35 containing 150 mM NaCl. Five changes of the dialysate was found to be enough to remove the unreacted material. The determination of the concentration of bound eosin was obtained by measuring the optical density at 528 nm using an extinction coefficient of 70,000 /M cm.

3. Reaction of Thyroxine (T4) with Double-Labeled T4 Antibody

The reaction of antigen with double-labeled antibody took place in 2 mL of a 5 mM sodium phosphate buffer containing 0.15 M NaCl (pH 8.0) inside a spectrophotometric cell (cuvette) after 1-2 minutes of incubation at room temperature.

4. Results

As can be seen in Figure 1, the presence of thyroxine reduced the fluorescence excitation transfer between the two chromophores. Figure 2 demonstrates that increasing volumes of a 1 mg/ml concentration of thyroxine produced a concentration dependent decrease of the relative fluorescence of the double-labeled antibody when measured at peak fluorescent intensity. At the concentrations used, this assay was linear. No significant changes in fluorescence were observed with equal volumes of vehicle solution or BSA (1 mg/ml).

EXAMPLE II

Assay for Hepatitis B Surface Antigen (HBsA)

1. Preparation of Double Labeled Antibody to HBsA

Material was obtained from Nuclear Medicine Laboratories in a kit form (NML*HBsAg RIA). Fluorescein and eosin labeling of the antibody to HBsA was accomplished using essentially the same met ods set forth in Example I for the T4 assay.

2. Reaction of HBsA to Anti-HBsA

The reaction of antigen with double-labeled antibody took place in 2 mL of a 5 mM sodium phosphate buffer containing 0.15 M NaCl (pH 8.0) inside a spectrophotometric cell (cuvette) after 1-2 minutes of incubation at room temperature.

3. Results

As can be seen in Figure 3, increasing amounts of HBsA produced a concentration dependent decrease of the relative fluorescence of double-labeled antibody to HBsA when measured at peak fluorescence intensity.

EXAMPLE III

Immunoglobulin G (IgG) Assay

1. Preparation of Double-Labeled Antibody to IgG

The materials were obtained commercially and labeling was performed essentially as described in Example I for the T4 assay.

2. Reaction of IgG to Anti-IgG

The reaction of antigen with double-labeled antibody took place in 2 mL of a 5 mM sodium phosphate buffer containing 0.15 M NaCl (pH 8.0) inside a spectrophotometric cell (cuvette) after 1-2 minutes of incubation at room temperature.

3. Results

In a manner similar to that observed in the two prior assays, increasing amounts of IgG decreased the relative fluorescence of the double-labeled antibody when measured at peak fluorescence intensity (see Figure 4).

EXAMPLE IV

Digoxin Assay

1. Preparation of Double-Labeled Digoxin Antibody

The materials were obtained commercially and digoxin antibody was double-labeled essentially as described in Example I for the T4 assay.

2. Reaction of Digoxin to Anti-Digoxin

The reaction of antigen with double-labeled antibody took place in 2 mL of a 5 mM sodium phosphate buffer containing 0.15 M NaCl (pH 8.0) inside a spectrophotometric cell (cuvette) after 1-2 minutes of incubation at room temperature.

3. Results

Unlike the above assays, incubation of double-labeled digoxin antibody with digoxin increased the fluorescence excitation transfer (see Figure 5). As can be seen in Figure 6, increasing amounts of digoxin produced a concentration dependent increase in relative fluorescence when measured at peak fluorescence intensity.

EXAMPLE V

IgG Measurement in Human Serum

IgG was measured in human serum by combining a small volume of serum (50-250 µl) with a detergent (e.g., sodium dodecylsulfate (SDS)) so that the final concentration of the SDS was about 10%. This mixture was added to 5 mM sodium phosphate buffer containing 0.15 M NaCl (pH 8.0) inside a spectrophotometric cell (cuvette) after 1-2 minutes of incubation at room temperature, and the assay completed as described in Example I. A graph displaying the ability to measure IgG in human serum samples is depicted in Fig. 7.

CLAIMS

What is claimed is:

1. A member of a specific binding pair of ligand and receptor, said member being covalently bound to first and second fluorophores, the first of said fluorophores being capable of absorbing light energy at a first wavelength to produce light energy emission at a second wavelength, which second wavelength can be absorbed by the second fluorophore, wherein specific binding of ligand and receptor affects energy transfer between said fluorophores.

2. The member of claim 1 wherein the second fluorophore produces an emission of light at a third wavelength upon absorbing light of the second wavelength.

3. The member of claim 1 wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

4. The member of claim 2 wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

5. The member of claim 1 wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

6. The member of claim 2 wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

7. The member of claim 1 wherein said second fluorophore absorbs light at said second wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

8. The member of claim 2 wherein said second fluorophore emits light at said third wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore and light emission by said second fluorophore at said third wavelength.

9. The member of claim 1 wherein said second fluorophore absorbs light at said second wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

10. The member of claim 2 wherein said second fluorophore emits light at said third wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore and light emission by said second fluorophore at said third wavelength.

11. The member of claim 1 wherein said member is receptor and said receptor is antibody to said ligand.

12. The member of claim 2 wherein said member is receptor and said receptor is antibody to said ligand.

13. The member of claim 8 wherein said member is receptor and said receptor is antibody to said ligand.

14. The member of claim 10 wherein said member is receptor and said receptor is antibody to said ligand.

15. The member of claim 8 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

16. The member of claim 10 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

17. The member of claim 11 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

18. The member of claim 12 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

19. The member of claim 8 wherein said ligand is Digoxin.

20. The member of claim 10 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.

21. The member of claim 11 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, Thyroxin or Digoxin.

22. The member of claim 12 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, Thyroxin or Digoxin.

23. The member of claim 13 wherein said ligand is Digoxin.

24. The member of claim 14 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.

25. The member of claim 15 wherein said ligand is Digoxin.

26. The member of claim 16 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.

27. A method for determining the presence or amount of a first member of a specific binding pair of ligand and receptor in a fluid sample, the method comprising:

(a) forming a reaction mixture by contacting said sample with a second member of said pair, said second member being covalently bound to first and second fluorophores, the first of said fluorophores being capable of absorbing light at a first wavelength to produce light emission at a second wavelength, which second wavelength can be absorbed by the second fluorophore;

(b) irradiating the reaction mixture with light of the first wavelength; and

(c) measuring the amount of fluorescence from the reaction mixture as compared to a standard.

28. The method of claim 27 wherein the second fluorophore produces an emission of light at a third wavelength upon absorbing light of the second wavelength.

29. The method of claim 27 wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

30. The method of claim 28 wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

31. The method of claim 27 wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

32. The method of claim 28 wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

33. The method of claim 27 wherein said second fluorophore absorbs light at said second wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore.

34. The method of claim 28 wherein said second fluorophore emits light at said third wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair inhibits absorption by the second fluorophore of light emitted by the first fluorophore and light emission by said second fluorophore at said third wavelength.

35. The method of claim 27 wherein said second fluorophore absorbs light at said second wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore.

36. The method of claim 28 wherein said second fluorophore emits light at said third wavelength upon emission of light of said second wavelength by said first fluorophore in the absence of binding of the pair, and wherein specific binding of the pair enhances absorption by the second fluorophore of light emitted by the first fluorophore and light emission by said second fluorophore at said third wavelength.

37. The method of claim 27 wherein said second member is receptor and said receptor is antibody to said ligand.

38. The method of claim 28 wherein said second d member is receptor and said receptor is antibody to said ligand.

39. The method of claim 34 wherein said second member is receptor and said receptor is antibody to said ligand.

40. The method of claim 36 wherein said second member is receptor and said receptor is antibody to said ligand.

41. The method of claim 34 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

42. The method of claim 36 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

43. The method of claim 37 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

44. The method of claim 38 wherein the first fluorophore is fluorescein and the second fluorophore is eosin.

45. The method of claim 34 wherein said ligand is Digoxin.

46. The method of claim 36 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.

47. The method of claim 37 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, Thyroxin or Digoxin.

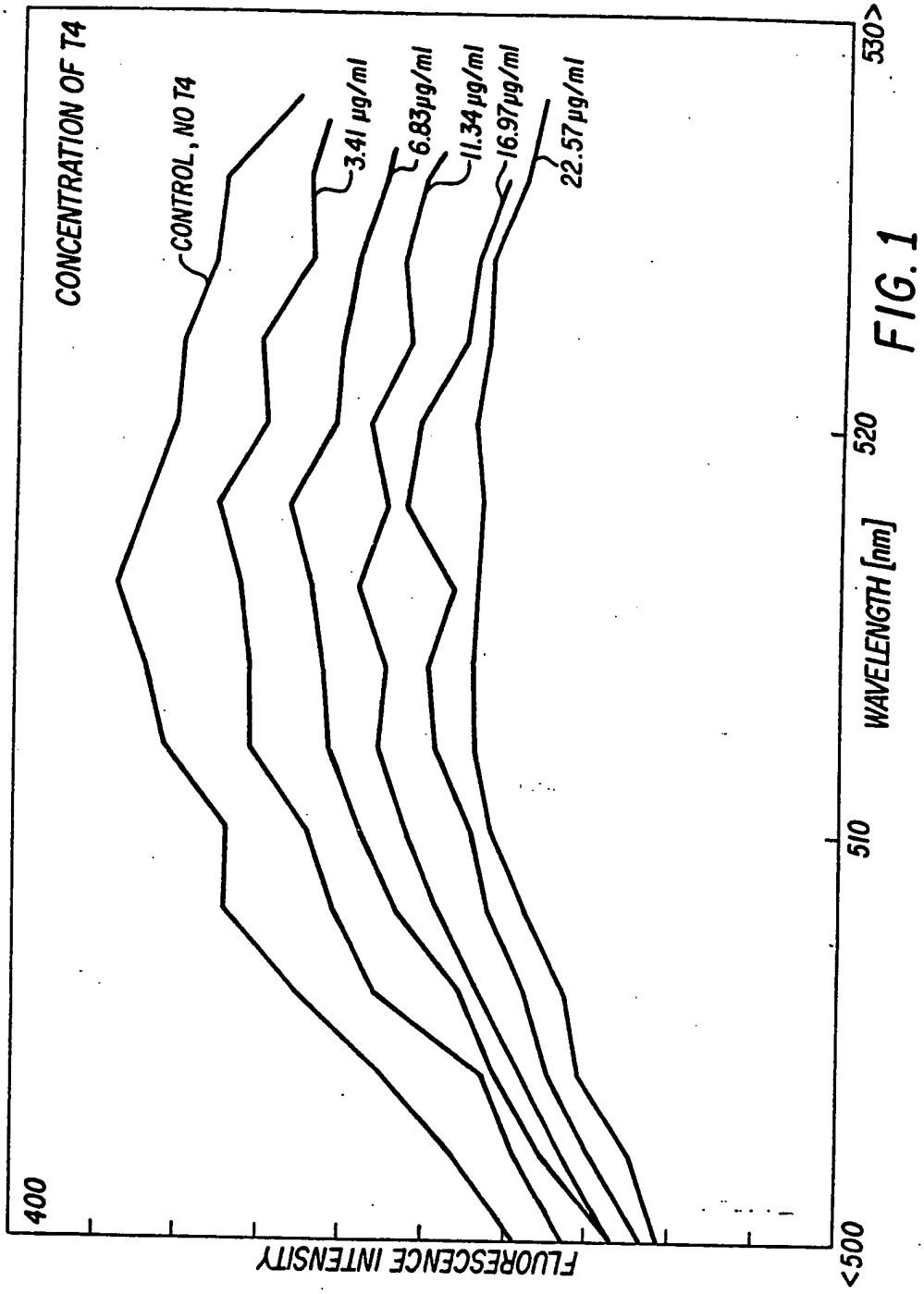
48. The method of claim 38 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, Thyroxin or Digoxin.

49. The method of claim 39 wherein said ligand is Digoxin.

50. The method of claim 40 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.

51. The method of claim 41 wherein said ligand is Digoxin.

52. The method of claim 42 wherein said ligand is Hepatitis B Surface Antigen, Immunoglobulin G, or Thyroxin.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400
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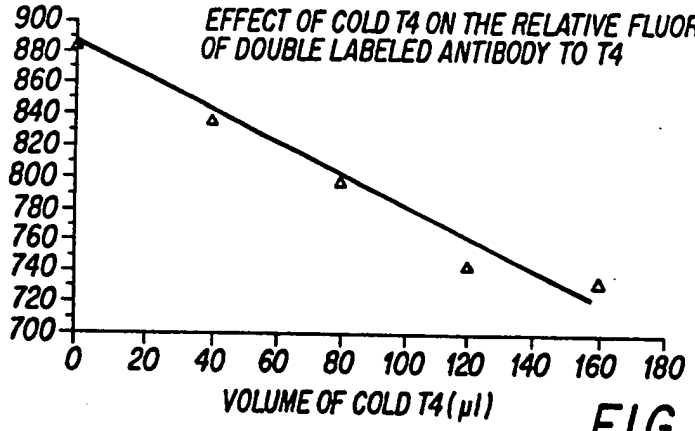
510
520
530>
FLUORESCENCE INTENSITY
WAVELENGTH [nm]

FIG. 1



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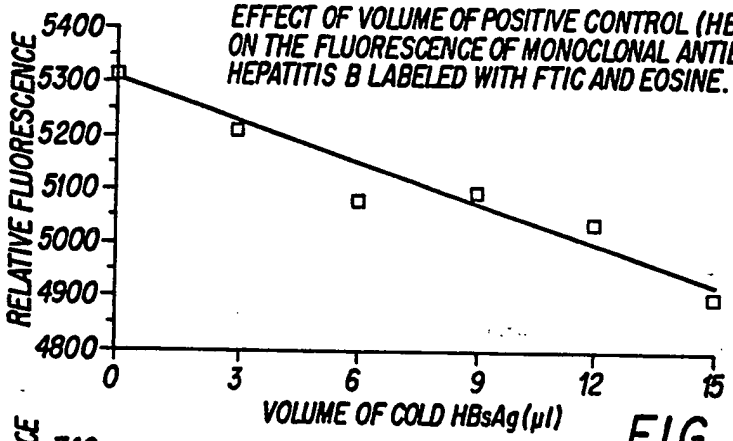


FIG. 3

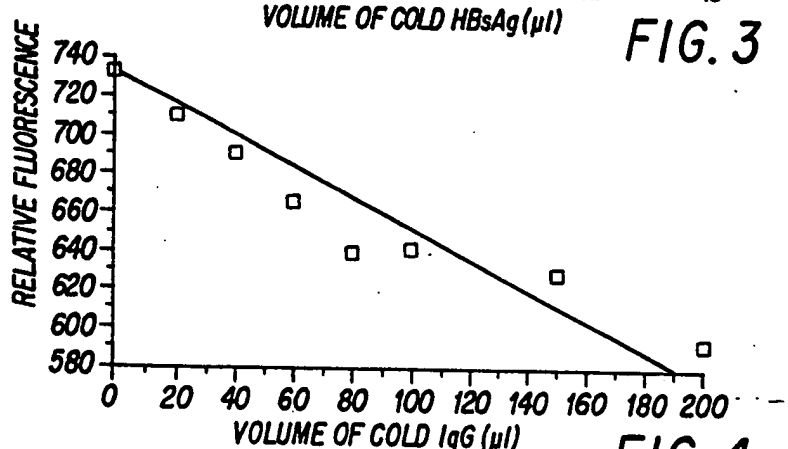
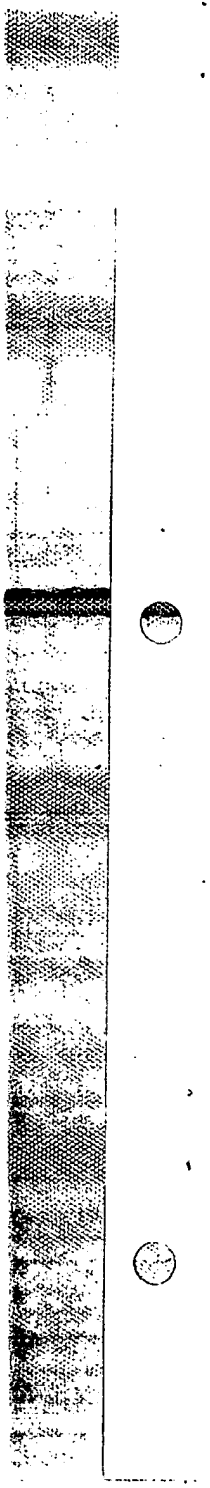


FIG. 4



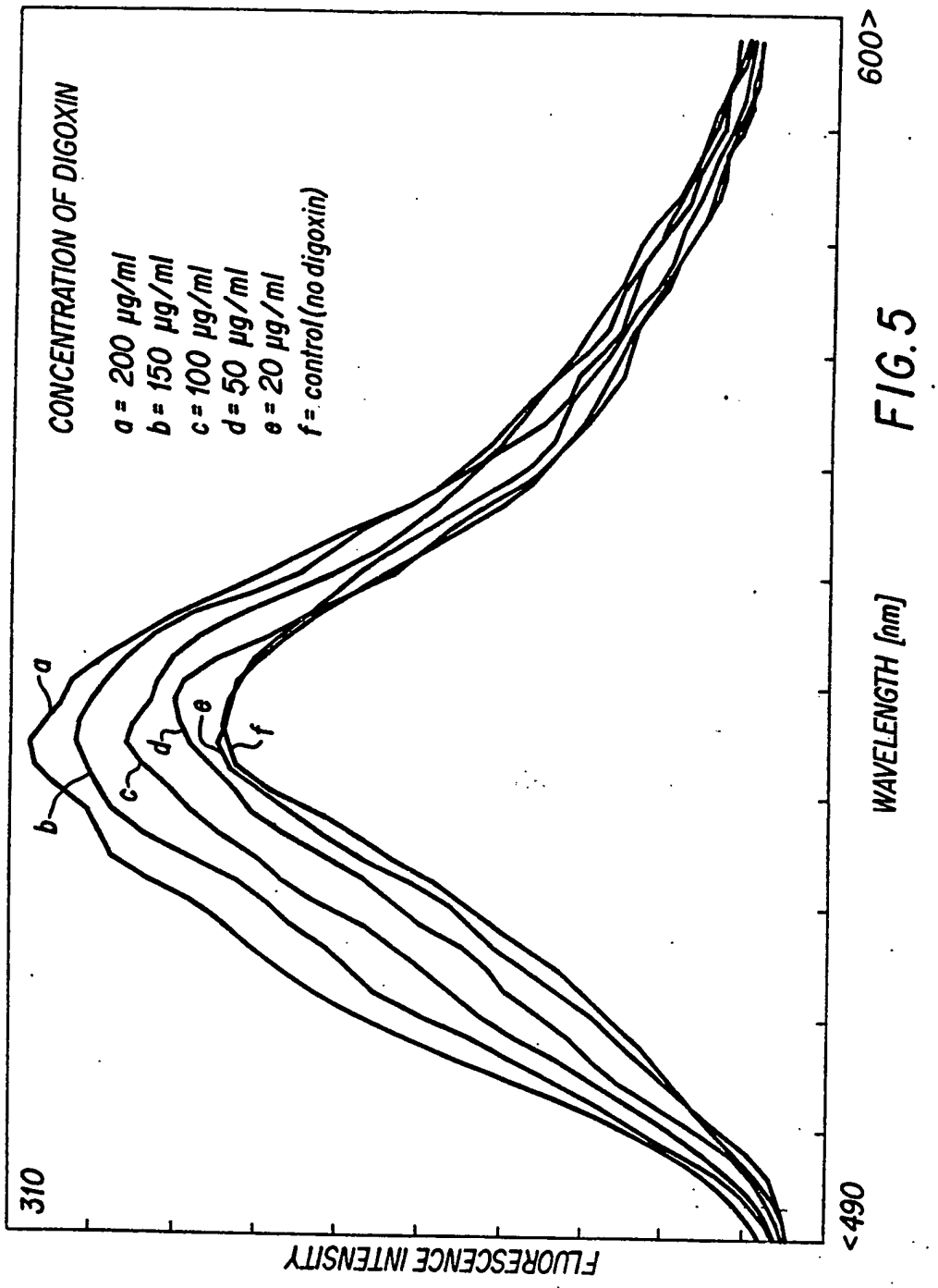

CONCENTRATION OF DIGOXIN
a = 200 µg/ml
b = 150 µg/ml
c = 100 µg/ml
d = 50 µg/ml
e = 20 µg/ml
f = control(no digoxin)
a
b
c
d
e
f
310
<490
600>
WAVELENGTH [nm]
FLUORESCENCE INTENSITY

FIG. 5

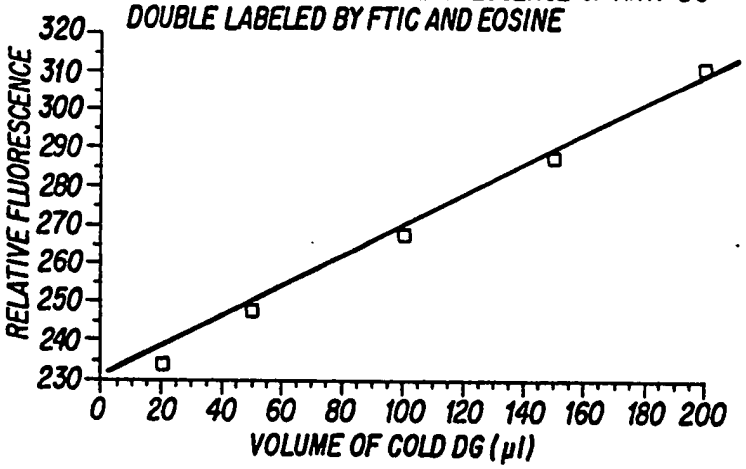

EFFECT OF COLD DG ON FLUORESCENCE OF ANTI-DG DOUBLE LABELED BY FTIC AND EOSINE
RELATIVE FLUORESCENCE
320
310
300
290
280
270
260
250
240
230
0
20
40
60
80
100
120
140
160
180
200
VOLUME OF COLD DG (µl)

FIG. 6

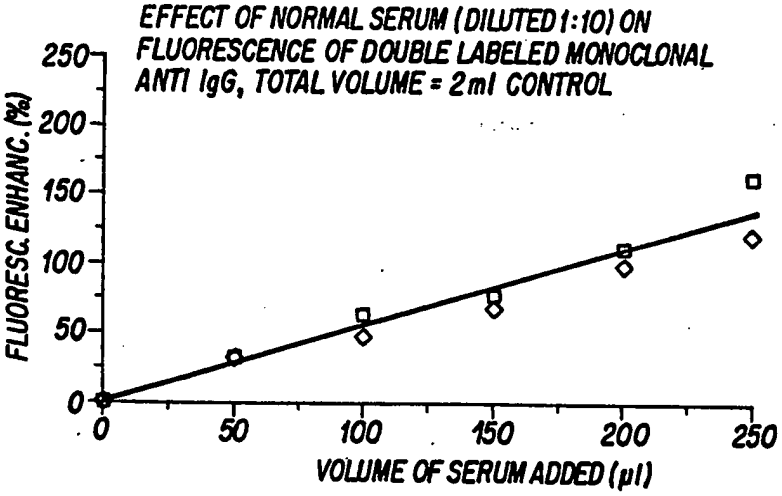

EFFECT OF NORMAL SERUM (DILUTED 1:10) ON FLUORESCENCE OF DOUBLE LABELED MONOCLONAL ANTI IgG, TOTAL VOLUME = 2ml CONTROL
FLUORESC. ENHANC. (%)
250
200
150
100
50
0
0
50
100
150
200
250
VOLUME OF SERUM ADDED (µl)

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01201

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/533, 542,571
U.S. Cl.: 436/501, 537, 800

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System 5	Classification Symbols
U.S.	436/172, 501, 537, 800

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 6

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
A	U.S., A, 4,261,968 (ULLMAN ET. AL.) 04 APRIL 1981. See entire document.	
A	U.S., A, 4,385,126 (CHEN ET. AL) 24 MAY 1983. See column 3, line 35 - column 4, line 41.	
X	U.S., A, 4,499,052 (FULWYLER) 12 FEBRUARY 1985. See examples 1 and 2.	1-14
Y	U.S., A, 4,536,479 (VANDER-MALLIE) 20 AUGUST 1985. See example 4.	15-18,25, 26,41-44,51 and 52
X Y	U.S., A, 4,542,104 (STRYER ET. AL.) 17 SEPTEMBER 1985. See column 1, line 50 - column 3, line 48.	1-14 and 27-40 15-26 and 41-52.
X,P Y,P	U.S., A, 4,666,862 (CHAN) 19 MAY 1987. See column 4, line 63 - column 6, line 15.	1-14 and 27-40 15-26 and 41-52

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
29 JULY 1987	11 AUG 1987
International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	David A. Saunders